Characterization of the ECB Binding Complex Responsible for the M/G₁-Specific Transcription of CLN3 and SWI4

Bernard Mai,† Shawna Miles, and Linda L. Breeden*

Fred Hutchinson Cancer Research Center, Division of Basic Sciences, Seattle, Washington 98109-1024

The transcription factor Mcm1 is regulated by adjacent binding of a variety of different factors regulating the expression of cell-type-specific, cell cycle-specific, and metabolic genes. In this work, we investigate a new class of Mcm1-regulated promoters that are cell cycle regulated and peak in late M-early G₁ phase of the cell cycle via a promoter element referred to as an early cell cycle box (ECB). Gel filtration experiments indicate that the ECB-specific DNA binding complex is over 200 kDa in size and includes Mcm1 and at least one additional protein. Using DNase I footprinting in vitro, we have observed protection of the ECB elements from the CLN3, SWI4, CDC6, and CDC47 promoters, which includes protection of the 16-bp palindrome to which Mcm1 dimers are known to bind as well as protection of extended flanking sequences. These flanking sequences influence the stability and the variety of complexes that form on the ECB elements, and base substitutions in the protected flank affect transcriptional activity of the element. Chromatin immunoprecipitations show that Mcm1 binds in vivo to ECB elements throughout the cell cycle and that binding is sensitive to carbon source changes.

Mcm1, its mammalian counterpart (serum response factor), and other members of the MADS box family of transcription factors share a 56-amino-acid MADS box, carrying a conserved DNA binding and dimerization domain (42). They are combinatorial transcription factors in that they typically bind adjacent to and derive their regulatory specificity from other DNA binding and/or accessory factors (3, 20, 42). Figure 1 depicts four classes of transcription complexes that include Mcm1. Depending upon the context, Mcm1 can be a component of an activating or a repressing complex, and these complexes can be regulated by specific events in the cell cycle or in response to internal or external cues. For example, repression of **a**-specific genes in α haploid and **a**/α diploid cells involves cooperative binding of α2 and Mcm1 to adjacent binding sites (22, 39). Then α2 recruits the Ssn6/Tup1 repressor complex to the site and prevents transcription (16). In contrast, α-specific genes are induced by Mcm1 and another α-specific transcription factor, α1 (43). In **a** cells, α2 is not produced, so the repressing complex cannot be formed. In that case, Mcm1 cooperates with another haploid-specific transcription factor, Ste12, to activate transcription and confer pheromone responsiveness to these promoters (12, 14).

Mcm1 is also involved in the regulation of arginine metabolism (not shown), where it forms a complex with Arg82 and two other DNA binding proteins: Arg80 and -81 (13, 33). The presence of arginine is sensed by Arg82 and results in the activation of transcription of catabolic enzymes and repression of anabolic enzymes, depending upon the context. Arg82 has recently been identified as an inositol-1,4,5-triphosphate ki-

nase, and this discovery suggests a direct connection between the lipid signaling cascade and gene regulation in yeast (35).

Mcm1 also plays a role in two consecutive waves of transcription during M phase and at the M/G₁ boundary of the cell cycle. The M-specific genes have a binding site for Mcm1 adjacent to a binding site for a forkhead transcription factor (Fkh1 or Fkh2) (23, 25, 27, 41, 54). Mcm1 and Fkh2 are bound to adjacent sites in the promoters throughout the cell cycle, but the association of the Ndd1 protein to the complex is correlated with and required for the activation of transcription. There is also evidence of transient phosphorylation of Fkh2 (41), but the significance of this modification has not been determined.

The M/G₁-specific transcripts are the most recently identified class of Mcm1-regulated transcripts. A promoter element, referred to as an early cell cycle box (ECB), which confers M/G₁-specific transcription was first identified in the SWI4 promoter. Then highly related sequences were identified in the promoters of four other M/G₁-specific transcripts which encode Cln3, Cdc6, Cdc46 (Mcm5), and Cdc47 (Mcm7) (32). The ECB includes a binding site for Mcm1, which was shown to be required for its activity. This observation provided a plausible explanation for why Mcm1 was initially identified among a collection of mutants that were defective in minichromosome maintenance (39). CDC6, whose product nucleates the formation of the preinitiation complexes on origins of replication (9, 46), requires Mcm1 for its transcription (32). Moreover, expression of CDC6 from a heterologous promoter suppresses the Mcm phenotype of an mcm1 mutant (32). This suggests that the Mcm phenotype of mcm1 mutants is an indirect effect of Mcm1's role in CDC6 transcription.

The ECB element includes a 16-bp palindrome to which Mcm1 is known to bind (3, 20, 39). Moreover, a fusion of the DNA binding and dimerization domain of Mcm1 to the VP16 activation domain can provide the essential function of Mcm1 but results in a low constitutive level of transcription of CDC6,

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, Division of Basic Sciences, 1100 Fairview Ave. N., Seattle, WA 98109-1024. Phone: (206) 667-4484. Fax: (206) 667-6526. E-mail: lbreeden@fhcrc.org.
† Present address: Aventis Pharma GmbH, Center for Functional Genomics, Fraunhoferstr. 13, D-82152 Martinsried, Germany.
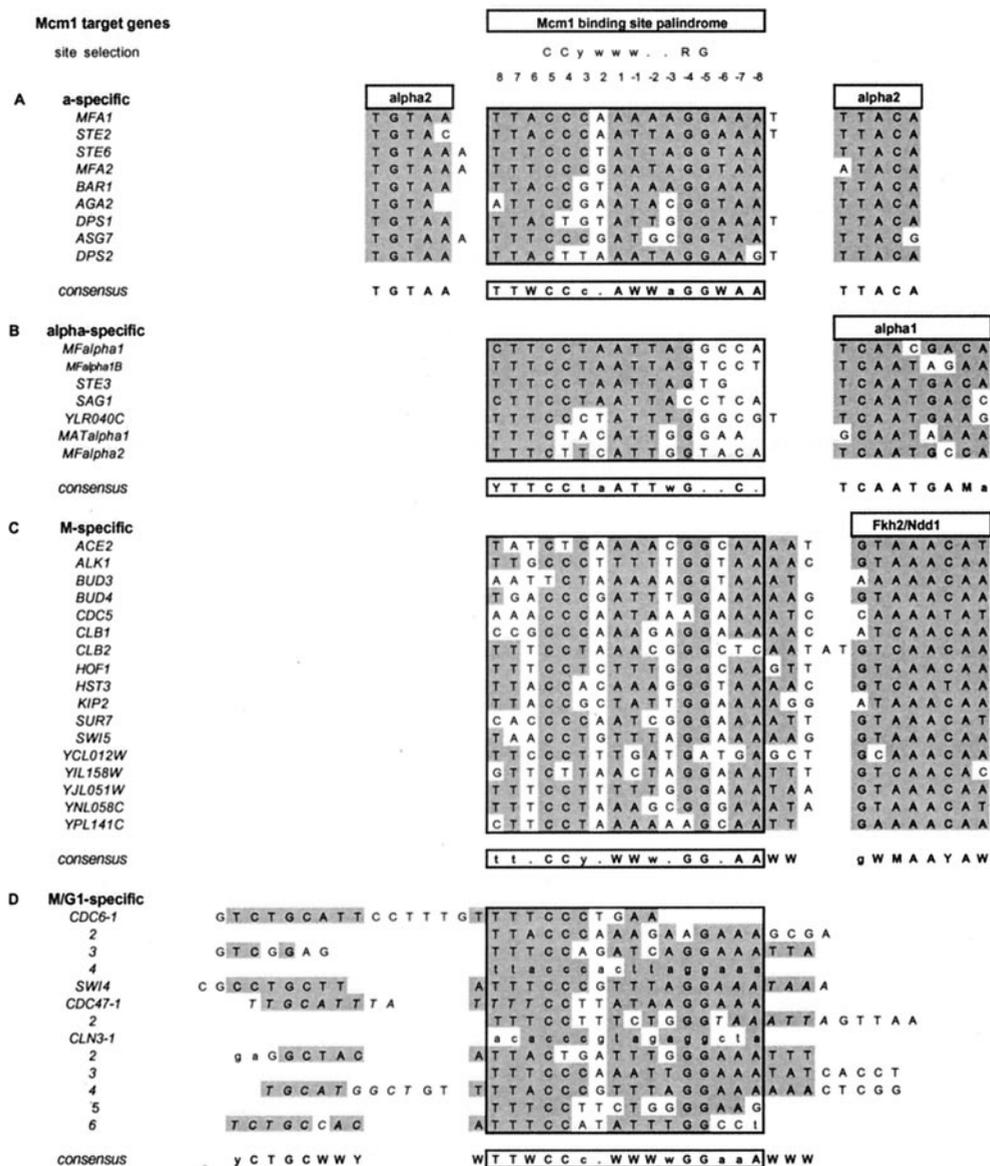
FIG. 1. Binding sites of Mcm1-containing complexes. Compilation of Mcm1 binding sites in the promoters of four different classes of Mcm1 target genes based on previous studies (32, 44, 52, 54). The minimal Mcm1 binding site identified by site selection is shown at the top. Below are the in vivo binding sites for four different classes of Mcm1 target genes, and the 16-bp Mcm1 binding site is boxed. Positions fitting the consensus sequence for each group are shaded, and the consensus is shown below. Bases written in uppercase depict those which are more than 75% conserved; bases in lowercase show positions identical in at least half the target genes. W = A or T, K = G or T, M = A or C, Y = C or T, R = A or G, and a dot indicates any base. Boxes above each flanking homology indicate the proteins which are known to bind to those sites and confer regulatory specificity to the complex. The M/G_1-specific genes are shown in section D, and all the sequences that are protected from DNase I cleavage (see Fig. 2 and 3) are shown in capital letters. Other potential ECB elements and a few other residues that are not protected from DNase I are shown in lowercase for purposes of comparison. Italic letters indicate that footprints were obtained only on the opposite strand to that shown. The sequence flanking the Mcm1 binding site in the M/G_1-specific promoters is aligned to show a region of limited sequence homology that is protected from DNase I cleavage.

CLN3, and *SWI4* (32). Unlike the other known Mcm1 binding sites, the ECB elements do not include an obvious binding site for a second regulatory factor. In fact, the two tandem 16-bp palindromes from the *CDC47* promoter were cloned into a reporter construct and were shown to be sufficient to confer M/G_1-specific transcription within that context (32). Thus, evidence has been lacking for the existence of accessory proteins or adjacent promoter elements that may be responsible for or contribute to the M/G_1 specificity of ECB complexes.

This work characterizes ECB elements in the promoters of four M/G_1-specific genes. We have investigated the in vitro and in vivo association of Mcm1 with these elements by gel retardation, DNase I footprinting, and chromatin immunoprecipitation (CHIP) experiments. We find that Mcm1 binds in vitro

and in vivo to all ECB-containing promoters tested but that some potential ECB sites are not bound. ECB sequences are bound by Mcm1 throughout the cell cycle in vivo, and the extent of binding is influenced by the carbon source. Gel filtration experiments indicate that ECB binding complexes are larger than expected for an Mcm1 homodimer binding alone. In addition, we observe protection from DNase I cleavage that extends beyond the l6-bp palindrome for about 10 bp in one or both directions. These flanking regions influence the complex formation and in vivo activity of the ECB elements.

MATERIALS AND METHODS

Yeast strains. All strains are derivatives of W303a (**a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3 can1-100 ssd1-d*). BY2278 (*cln3ecb-5*) and BY2690 (*cln3ecb-6*) contain mutations in five or six of the potential CLN3 ECB sequences, respectively, and have been previously described (28). Cells were grown in yeast extract-peptone (YEP) media supplemented with 2% carbon source as indicated.

CHIP. Chromatin-containing whole-cell extracts were prepared as previously described (47), with some modifications. Yeast cells (50 ml, 3×10^7 cells/ml) were cross-linked with 1% formaldehyde (final concentration) for 15 min at room temperature. After addition of 125 mM glycine and incubation for 5 min, cells were harvested and washed once with phosphate-buffered saline. Cell breakage was performed in 500 μl of lysis buffer {50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Na deoxycholate, 1 μg of pepstatin A/ml, 1 μg of leupeptin/ml, and 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride · HCl]} with glass beads using a Bead Beater (FastPrep120; Q-Biogene, Carlsbad, Calif.) at level 4.5 for 40 s. Whole-cell extracts were sonicated for 30 s on level 7 (Sonifier Cell Disruptor; Heat Systems). The protein concentration in the soluble chromatin extract was determined using a Bradford assay. One milligram of protein and 8 μl of crude rabbit antiserum directed against an Mcm1 peptide (20) were used for each immunoprecipitation (a generous gift from George Sprague). Precipitates were washed two times with lysis buffer, two times with lysis buffer containing 500 mM NaCl, two times with wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na deoxycholate, and 1 mM EDTA), and finally two times with Tris-EDTA. Bound complexes were eluted twice with 75 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% sodium dodecyl sulfate) incubated for 5 min at 65°C. Eluates were combined and cross-links were reversed by incubation at 65°C for 6 to 12 h. The DNA was purified using the QIAquick PCR purification kit and was eluted into 100 μl of water. For input samples, 10 to 20 μg of chromatin extract was combined with 140 μl of elution buffer, the cross-links were reversed, and the DNA was purified as described above.

PCR analyses and primers. PCRs were carried out in a 25-μl volume with 1/100 of the precipitated DNA or the input samples. *Taq* polymerase (Fisher) and the corresponding buffer system were used. PCR primers were designed to be 24 bases long with approximately 50% GC content. Sequences of PCR primers used are available upon request. Three or four pairs of primers were used in each PCR at a final concentration of 1 μM. PCR cycles involved an initial denaturation of 3 min at 95°C, followed by 25 cycles with 30 s of denaturation at 95°C, 20 s of annealing at 60°C, polymerization at 72°C for 1 min, and a final extension for 4 min at 72°C. PCR products were separated on 6% native polyacrylamide gels run in 1× Tris-borate-EDTA and were stained with ethidium bromide. The gel was photographed with a charge-coupled device camera. For quantification, gels were scanned with a fluorimeter (Molecular Dynamics) and band intensities were determined using ImageQuant software. The immunoprecipitated fraction was normalized to the input levels.

DNA manipulations. ECB-containing DNA fragments for the footprinting analyses were generated by PCR. Oligonucleotides containing either *Eco*RI or *Bam*HI restriction sites on the ends were designed to amplify 150 to 250 bp of promoter sequence from genomic DNA using a mixture of *Taq*-DNA polymerase and a proofreading polymerase. PCR products were cloned into pBluescript II KS(−) and confirmed by sequencing. The following plasmids were generated in this way: SWI4-ECB (BD2543), CLN3-ECB-1-4 (BD2544), CLN3-ECB-5 (BD2546), CLN3-ECB-6 (BD2547), CDC6-ECB (BD2545), and CDC47-ECB (BD2548). For DNase I footprinting analyses, 10 μg of plasmid DNA was cut with *Hin*dIII-*Sac*I or *Kpn*I-*Bam*HI to label the upper or lower strand of each fragment.

DNase I footprinting. Fragments were labeled at the 3′ end using the Klenow fragment of DNA polymerase and α-[^{32}P]dCTP. After purification through a 5% native polyacrylamide gel, fragments were used as probes in footprinting assays.

In a total volume of 20 μl, 15,000-cpm fragments were combined with partially purified Mcm1 in DNase I binding buffer [20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 5% glycerol, and 200 μg of poly(dIdC)/ml]. After 10 min of incubation at room temperature, 1 U of RNase-free DNase I (Roche) was added and incubated for 2 min. The reaction was stopped by addition of 100 μl of phenol. After vortexing, 80 μl of water and 100 μl of chloroform were added and the DNA was recovered by precipitation in the presence of 6 μg of glycogen. Samples were run on an 8% polyacrylamide–8 M urea gel, and bands were visualized by autoradiography. To orient the cleavage pattern relative to the sequence, a labeled DNA marker (pBR322; *Msp*I cut) was run in parallel.

Gel retardation assay. Gel retardation (or band shift) assays were performed as described (29) using crude cell extracts or fractions enriched for Mcm1 (see below). Binding reactions were performed using 20 fmol of labeled oligonucleotides in a total volume of 20 μl of binding buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM MgCl$_2$, 5% glycerol, and 25 μg of poly[dIdC]/ml). Complexes were separated on 5% polyacrylamide gels run at 180 V in 0.5× Tris-borate-EDTA buffer at room temperature.

Dissociation constants (K_d) were measured in gel retardation assays as described earlier (29), with a double-stranded, 39-bp oligonucleotide encompassing the fourth ECB element of CLN3 (GTGCATGGCTGTTTTACCCGTTTAGG AAAAAACTCGGCG) and compared to that of a second oligonucleotide with the same central 16-bp palindrome but with the flanking base substitutions italicized (*TCGATCGATCGAC*TTACCCGTTTAGGAAA*GACTGACTG*).

Enrichment for Mcm1. Yeast cells (typically 5 liters) were grown in YEP–2% glucose to an optical density at 600 nm of 1 to 2. Cells were collected by centrifugation, washed once with water, and resuspended in buffer B (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM MgCl$_2$, 5% glycerol, and the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin/ml, 1 μg of pepstatin A/ml, and 1 mM AEBSF). All subsequent steps were performed at 4°C. Cells were broken using a bead beater with 5 pulses of 45 s and cooling on ice in between. Extracts were clarified by centrifugation at $100,000 \times g$ for 45 min. At this point the extract (approximately 1 g of total protein) was frozen at −80°C or immediately loaded onto an SP Sepharose FF column (Pharmacia) (150-ml bed volume) equilibrated with buffer B. The column was washed with buffer B and eluted with a salt gradient (from 100 to 1,000 mM NaCl) in buffer B. Mcm1-containing fractions were eluted at a salt concentration of 400 to 550 mM NaCl. Peak fractions (approximately 100 mg of total protein) were combined, diluted by addition of an equal volume of buffer B (100 mM NaCl), and loaded onto a 5-ml HiTrap Heparin Sepharose column (Pharmacia). Mcm1-containing fractions eluted at 800 mM NaCl were assayed by band shift and Western blotting. Fractions showing peak binding activity to the CLN3 ECB probe (ggccGCATATTTCCAATTTGGGAAATTTCtcga, where lowercase indicates single-stranded 5′ overhanging bases) were combined, dialyzed against buffer B (100 mM NaCl), and stored at −80°C. This preparation contains many proteins and will be designated the Mcm1-enriched fraction.

The binding properties of Mcm1 were also analyzed using Mcm1 that was transcribed and translated in vitro using the TNT Reticulocyte Lysate system under the conditions specified by the vendor (Promega Corp., Madison, Wis.). Fifty-microliter reactions programmed with Mcm1 plasmid DNA were performed, and 1 μl of this mix was added to each gel retardation assay.

Gel filtration. Mcm1-enriched fractions or crude cell extracts, clarified by ultracentrifugation ($30,000 \times g$, 45 min, 4°C), were loaded at 1 ml/min onto a Sephacryl S200 column (16 by 60 mm) run at 4°C in binding buffer (without poly[dIdC]). One-milliliter fractions were collected according to the position of marker proteins in the size range of 250 kDa (exclusion limit) to 10 kDa. Fractions were analyzed by gel retardation assays and Western blots.

RNA measurements. Northern blotting was performed as described in reference 29 by using 10 μg of total yeast RNA per lane. S1 protection using oligonucleotide probes was carried out as described earlier (19, 30), except that the probes were purified over a G-25 Sephadex column and ethanol precipitated once with tRNA as the carrier and were then heated above 65°C for 10 min before hybridization.

RESULTS

The sequence responsible for the M/G$_1$-specific transcription of several cell-cycle-regulatory genes has been identified and designated the ECB (28, 32) to distinguish it from other Mcm1 binding sites. Figure 1 shows four classes of Mcm1-dependent promoters and the derived consensus binding sites for Mcm1 and the known accessory factors. These binding sites

contain the minimal consensus binding site for Mcm1 as identified by site selection (34), as well as a conserved 3-bp extension on either side that is conserved to differing extents in other Mcm1 binding sites. Mcm1 binds as a dimer to these sites and forms base-specific contacts spanning this 16-bp palindrome (48) that are required for in vivo activity (1). In addition to the Mcm1 binding site, there is typically a binding site for another transcription factor precisely positioned in the adjacent DNA which confers the regulatory specificity to the complex. The M/G$_1$-specific ECB elements show symmetrical and extensive conservation across the palindrome, but there is no extensive sequence conservation adjacent to it which would suggest a binding site for another protein. The aim of this study was to determine whether or not other proteins are involved in ECB function and to characterize the complexes in vitro and in vivo.

ECB binding complexes protect Mcm1 binding site and a flanking sequence from DNase I digestion. To study the extent of binding to ECB sequences in vitro, we used DNase I protection assays. For this purpose, end-labeled fragments from ECB-containing promoters of *CLN3*, *SWI4*, *CDC6*, and *CDC47* were digested with DNase I in the presence or absence of yeast protein extracts. We could not detect Mcm1 binding from yeast crude extracts by this assay, so an Mcm1-enriched fraction, in which the Mcm1-containing fractions were pooled following SP-Sepharose and heparin-Sepharose column chromatography, was used. The resulting protection pattern was compared to a pattern obtained with DNA in the absence of protein extract. As shown in Fig. 2 and 3, Mcm1 binds most putative ECB sequences, although clear differences in the protection pattern can be observed.

In the *CLN3* promoter there are six ECB-like sequences in the first 1,000 bp upstream of the ATG (Fig. 1). To assay binding to all of these sites, the promoter was split into three fragments and assayed separately by DNase I footprinting. As shown in Fig. 2, ECB sequences 2, 3, and 4 are clearly protected on both strands. The first potential ECB shows protection only over half the site and only on one strand. The fifth ECB sequence is not protected at all. The glucose response elements (GREs) (37), positioned between ECB-5 and ECB-6, are not protected by this Mcm1-enriched fraction of the cell extract. The sixth ECB in the *CLN3* promoter is protected only on one strand (Fig. 2). In addition, DNase I-hypersensitive sites are found in the middle of the Mcm1 core binding site in ECB-3, -4, and -6 (Fig. 2). The double ECB in the *CDC47* promoter shows clear protection on both strands of both sites, although DNase I-hypersensitive sites are formed at the middle position of ECB-2 only (Fig. 3). The *CDC6* promoter shows a long protected region which contains a half-site for Mcm1 binding. We have labeled this half-site ECB-1 for reference, but its function as an ECB has not been investigated. There are three more potential ECBs. ECB-2 and -3 are clearly protected, but ECB-4 is not protected, even though it has a perfect consensus sequence within the 16-bp palindrome. ECB-3 is only protected on the upper strand. There is also no protection observed in the region of a possible Swi5 binding site (11, 49), and only one of the late G$_1$-specific MCB elements is protected on one strand (53). The single ECB in the *SWI4* promoter is clearly protected on both strands with DNase I-hypersensitive sites as indicated (Fig. 3).

Figures 2B and 3B summarize the footprint data, showing the locations of the DNase I-protected sequences as bars and hypersensitive sites as dots. Interestingly, the footprint patterns are not perfectly correlated with the presence of Mcm1 binding sites. Some sites are not protected, and those that are protected show extended footprints beyond the palindrome. These extensions are strand specific and ECB sequence specific. The flanking sequences protected from DNase I cleavage are included in Fig. 1D and aligned to show the only stretch of homology that is evident within the flanking protected region. This sequence (YCTGCWWY) is a candidate binding site for an accessory protein; however, it differs from other known binding sites for Mcm1 accessory factors in that the sequence conservation is much less extensive and its distance from the Mcm1 binding site varies between 0 and 7 bp. Nevertheless, protection from DNase I cleavage extends over this sequence in all four of the M/G$_1$-specific promoters analyzed and suggests that the Mcm1 dimer may not be the only protein within the ECB complex.

To explore this possibility, we analyzed the complexes that form on the fourth *CLN3* ECB using a 39-bp DNA fragment that includes the protected flanking sequence. Figure 4, lane 1, shows the heterogeneous and somewhat variable array of complexes that form on this sequence from crude cell extracts as assayed by gel retardation. Most of these complexes include Mcm1, as indicated by the ability of an Mcm1-specific antibody to further retard their mobility in the gel (Fig. 4, lane 2). However, it is likely that other proteins are included in these complexes, because when the same DNA is incubated with in vitro-translated Mcm1, we find only one prominent band and some minor species (Fig. 4, lane 3). The prominent band comigrates with the lowest specific band shift complex that forms on the *CLB2* (M specific) and *STE2* (**a** specific) Mcm1 binding sites (data not shown) and probably represents the Mcm1 dimer bound to DNA. We then compared the complexes that form on the *CLN3* ECB (Fig. 4, lanes 5 and 6) to those that form on a second duplex of comparable length in which the flanking sequences were mutated (Fig. 4, lanes 7 and 8). It is clear that the high-molecular-weight complexes are most abundant when the flanking sequence is intact. In the absence of this flanking sequence, Mcm1 can still bind, as indicated by the prominent lower band, but higher-molecular-weight complexes are less evident. We have measured the dissociation constants for the wild-type and mutant complexes and find that there is a threefold difference (0.08 and 0.26×10^{-9} M, respectively). This difference is consistent with the possibility that sequences flanking the Mcm1 binding site contribute to the binding affinity of ECB complexes.

To investigate ECB function in vivo, we have characterized the single ECB element from the *SWI4* promoter. We have previously shown that this ECB is required for the M/G$_1$-specific transcription of *SWI4* (32). Figure 5A shows that a 39-bp segment of the *SWI4* promoter, including all of the protected ECB sequence, is sufficient to confer cell-cycle-regulated transcription to a *lacZ* reporter construct. This transcription is not evident in the first cycle after release from the arrest, but it clearly peaks in the second cycle about 10 min before the peak of the *CLN1* transcript (Fig. 5A). This is identical to the kinetics of wild-type *SWI4* and the three other ECB-regulated transcripts that have been analyzed (5, 28, 32).

CLN3
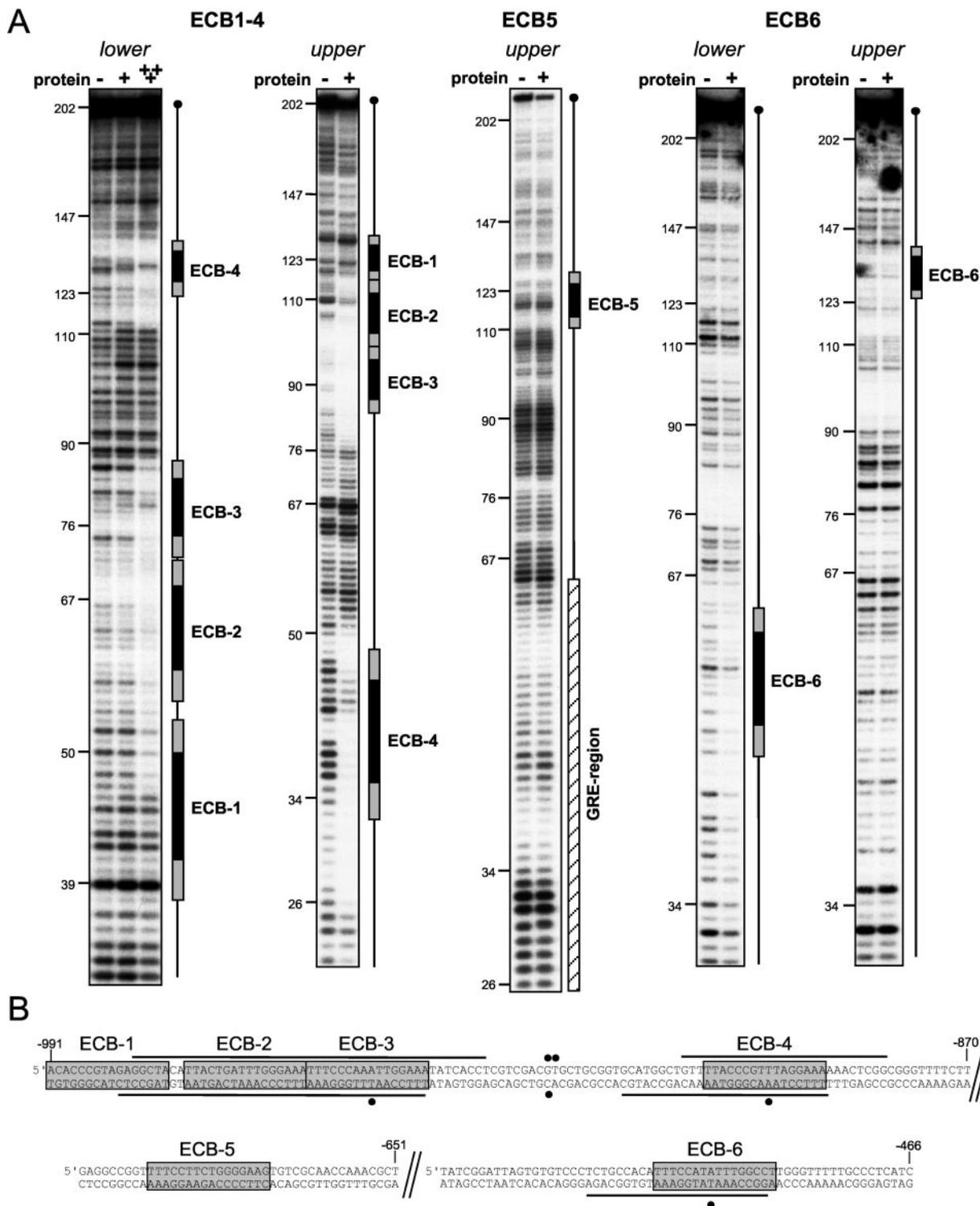
FIG. 2. DNase I footprints of partially purified Mcm1 binding to ECB-containing regions of the *CLN3* promoter. (A) Three different ECB-containing regions of the *CLN3* promoter were end labeled on one DNA strand. Upper and lower strand-specific probes (indicated at top) were used in binding reactions containing saturating amounts of Mcm1, which was partially purified from yeast extracts. After a 10-min incubation at 25°C, the probe was digested with DNase I for a limited time and purified, and digestion products were resolved on a 8% denaturing gel. *Msp*I-cut pBR322 was labeled, run in parallel, and used as a size marker. The positions and numbers of base pairs of the marker fragments are indicated to the left of each panel. ECB sequences are indicated with boxes, colored black for the position of the core Mcm1-consensus binding sequence (CCN$_6$GG) and gray for the A/T-rich 3-bp extensions. The hatched box depicts the region of the GREs. (B) Summary of the DNase I footprints. Positions of protected sequences (black lines) and DNase I-hypersensitive sites (black dots) are indicated in the sequence of the *CLN3* promoter. Numbers represent the distance in nucleotides to the *CLN3* ATG.
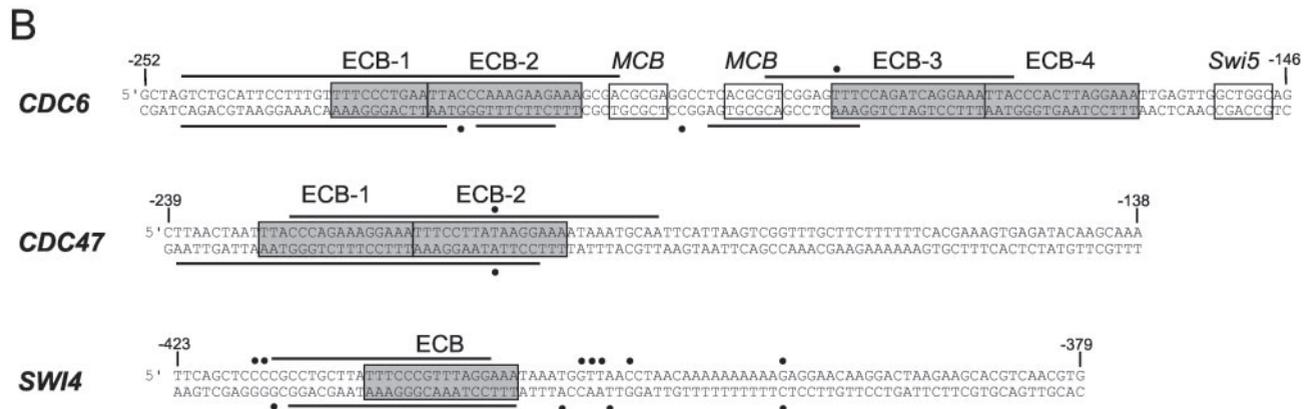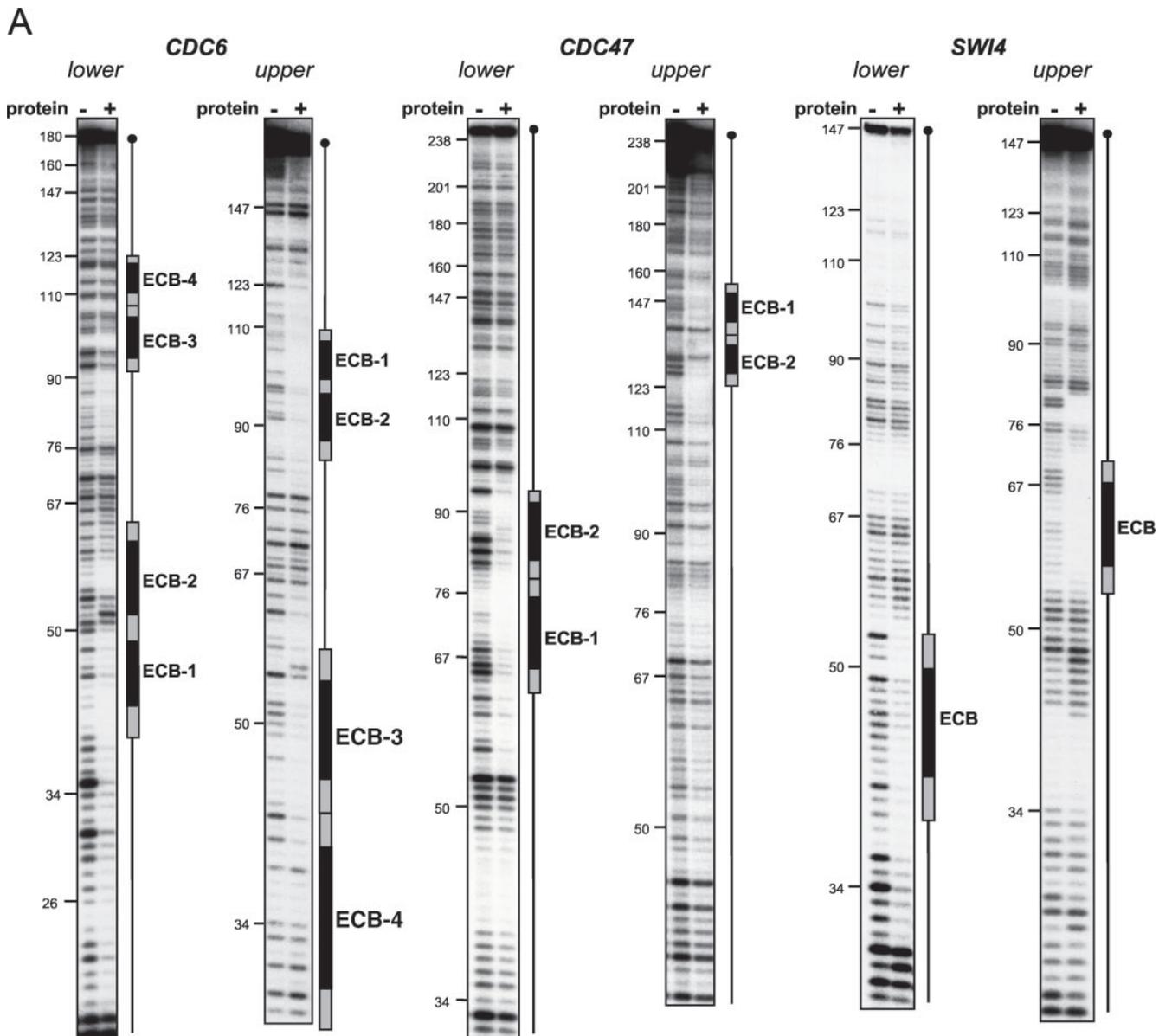
FIG. 3. DNase I footprints of partially purified Mcm1 binding to ECB-containing regions of the *CDC6*, *CDC47*, and *SWI4* promoters. (A) DNase I footprints were performed as described for Fig. 2 using strand-specific probes from the *CDC6*, *CDC47*, and *SWI4* promoters. (B) Summary of the DNase I footprints. The 16-bp palindromes of each ECB are indicated with gray boxes. The positions of protected sequences (black lines) and DNase I-hypersensitive sites (black dots) are indicated in the promoter sequences.
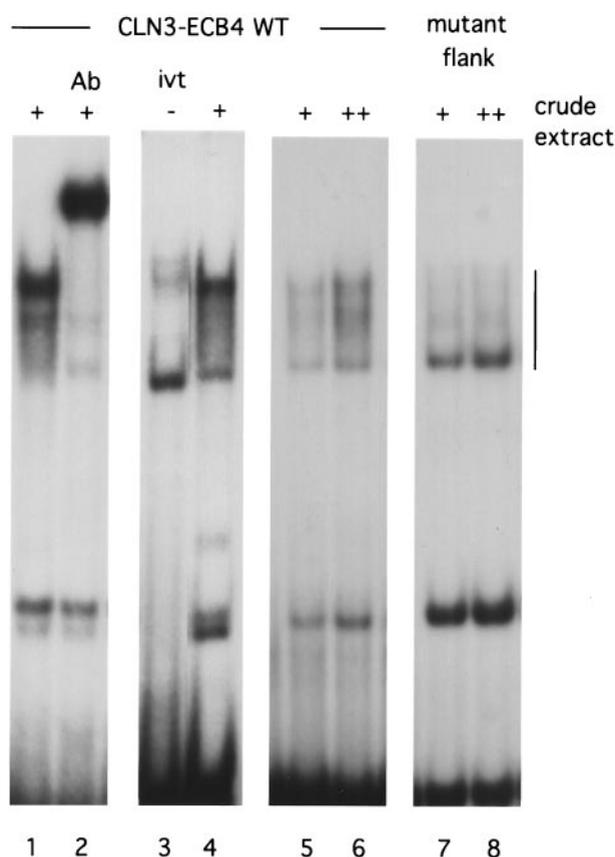
FIG. 4. Protein/DNA complexes on ECB elements include Mcm1 and are influenced by sequences flanking the Mcm1 binding site. Shown are gel retardation assays of complexes that form on a 39-bp DNA fragment, including all the sequences around the fourth *CLN3* ECB that are protected from DNase I cleavage (lanes 1 to 6) or those that form on the same palindromic site but lack the flanking sequences (lanes 7 and 8). Bar denotes Mcm1-specific complexes. All assays include 0.5 (+) or 1 (++) μl of crude cell extract, except lane 3, which shows the complexes formed with 1 μl of in vitro-translated Mcm1 (ivt). Polyclonal antiserum (Ab) directed against Mcm1 was added (0.5 μl) to lane 2.

It has previously been shown that M/G_1-specific transcription of *SWI4* requires Mcm1 binding activity (32). Similarly we find that disrupting the CCN_6GG core binding sequence for Mcm1 in the 39-bp fragment leads to a drop in *lacZ* activity to the level of the reporter with no promoter element inserted (data not shown). To see if the sequences flanking the Mcm1 binding site that are protected from DNase I digestion are also important for transcription, we analyzed the effect of mutating the most conserved residues in the flanking homology. Substitution of the conserved GC for CG led to an increase in the steady-state level of *lacZ* transcription to 140% ± 4%. When followed through the cell cycle, it is also evident that transcriptional activity of the mutant element is enhanced and possibly extended for a broader interval of time but that cell cycle regulation persists (Fig. 5B). At the peak of expression, this mutant induces almost twice as much mRNA as the wild-type element.

ECB binding activity migrates as large protein complex. The DNase I footprints extending beyond the 16-bp palindrome to which Mcm1 homodimers are known to bind reveals
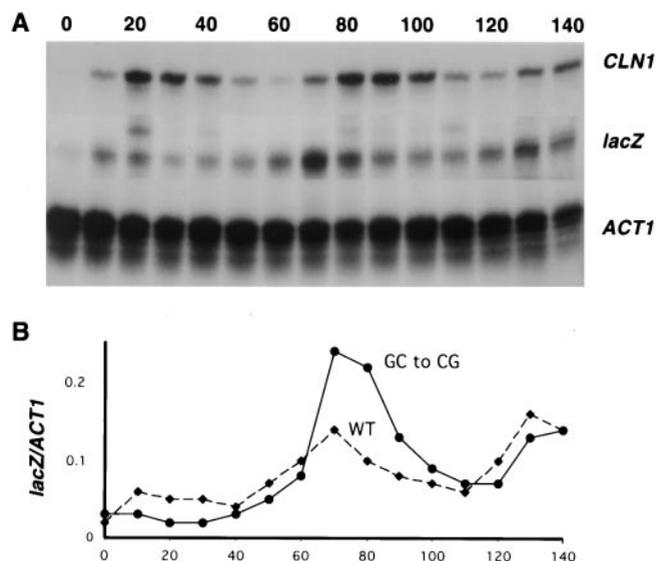
FIG. 5. Conserved bases in the protected flank influence the activity but not the cell cycle regulation of the *SWI4* ECB element. (A) S1 protection (see Materials and Methods) was used to monitor *CLN1*, *lacZ*, and *ACT1* mRNA levels through the cell cycle at 10-min intervals in α-factor-synchronized cells (7). The *lacZ* transcript is driven by a 39-bp fragment of the *SWI4* promoter containing the wild-type ECB plus flanking sequence. (B) Transcript levels were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.) The ratio of *lacZ* to the control RNA (*ACT1*) is plotted as a function of time over 2.5 cell cycles. The dashed line represents *lacZ* transcript driven by the wild-type (WT) ECB element, and circles show the transcript attained from a mutant ECB which has CG replacing the conserved GC in the flanking sequence. Both were integrated at the *URA3* locus of W303 and were assayed for transcriptional activity on the same day with the same radioactive probes.

the possibility that other proteins may bind to adjacent sites and interact with Mcm1 at ECB elements. To further characterize these ECB binding complexes, gel filtration experiments were performed. The partially purified Mcm1 used for the footprint studies was loaded onto a gel filtration column. Size-separated protein fractions were collected and assayed by band shift analysis for ECB-specific binding. In parallel, these fractions were assayed on Western blots for the presence of Mcm1 protein. As shown in Fig. 6A, fractions six and seven contain most of the Mcm1 protein. This position in the elution profile coincides with the exclusion limit of the column and indicates that Mcm1 is part of a complex of at least 200 kDa and possibly much larger. There is no Mcm1-specific signal detected at 70 or 35 kDa, which are the expected sizes of Mcm1 dimers and monomers. When the Mcm1-containing fractions were tested in band shift experiments for binding to a *CLN3*-ECB probe, the complexes detected migrated at a lower position in the gel than the complexes formed by the unfractionated extract (Fig. 6A). This suggests that some component of the ECB binding complex dissociated during the gel filtration. In order to test this hypothesis, the peak Mcm1 fraction (fraction 6) was combined with all other fractions of a gel filtration run. Interestingly, fractions containing proteins of 20 to 25 kDa (fraction 25) were able to restore the original ECB-specific complex (Fig. 6B). None of these fractions contained detectable Mcm1 protein or ECB binding activity (data not shown). Gel filtrations were
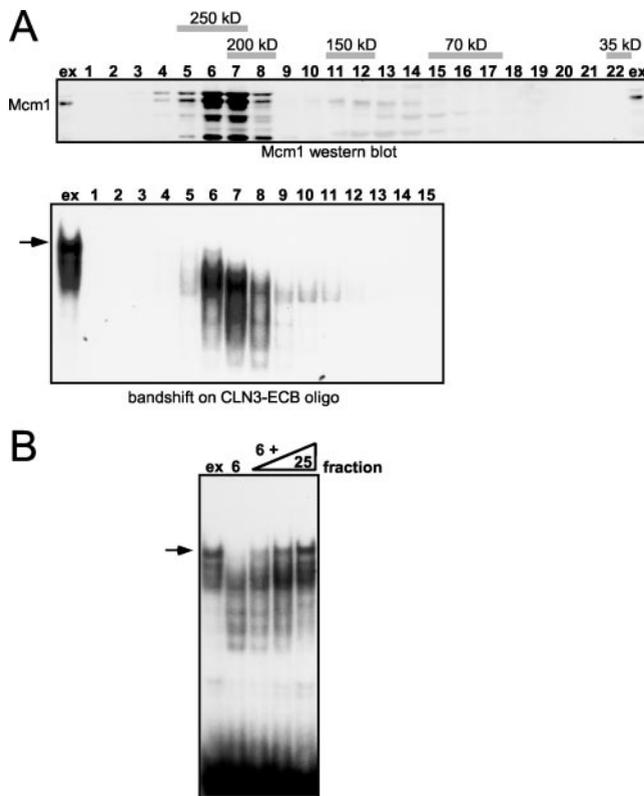
FIG. 6. Gel filtration analysis of ECB binding complexes. (A) Clarified yeast crude extracts were subjected to gel filtration on a Sephacryl S200 column. Fractions were collected and assayed by Western blotting for the presence of Mcm1 (upper panel) and by gel retardation assays for ECB binding activity (lower panel). The first lane contains the extract (ex) loaded onto the column. Numbers indicate fractions, and gray bars depict the elution position of marker proteins run in parallel. The band shift assay below shows the only fractions in which DNA binding complexes were detected. The arrow marks the ECB-specific complex formed from crude yeast cell extracts. (B) Reconstitution of the ECB-specific complex. The *CLN3*-ECB oligonucleotide was incubated with crude extract, fraction 6 of the gel filtration, or a combination of fraction 6 and later fractions from the same column. Addition of increasing amounts of fraction 25 (three right lanes) restored the complex to a position comparable to that obtained with crude cell extracts, denoted by the arrow.

FIG. 7. Mcm1 binds to ECB elements in vivo. (A) *CLN3* promoter region showing the position of the ECB (solid boxes) elements and the GREs (gray boxes). The positions of the PCR products generated with the *CLN3*-ECB primer sets (*CLN3*–ECB-a and *CLN3*–ECB-b) are indicated. (B) Sequence alignment of the six potential ECB elements of the *CLN3* promoter. Bases identical to the consensus (cons.) sequence are shaded. (C) Association of Mcm1 with *CLN3*-ECB- and *SWI4*-ECB-containing promoter regions in vivo. PCR products were separated on 6% native polyacrylamide gels run in 1× Tris-borate-EDTA and stained with ethidium bromide. PCR was performed on chromatin fragments isolated before (INPUT) and after (IP) immunoprecipitation with Mcm1 antibodies (αMcm1) or preimmune serum (PI) from whole-cell extract with or without (no X-link) prior formaldehyde cross-linking. The samples were prepared from BY2125 (W303a) grown in YEP glucose medium at 30°C. Lanes 7 and 8 show PCRs performed on input DNAs taken in threefold serial dilutions. (D) Association of Mcm1 with *CLN3*-ECB, *SWI4*-ECB, and *CLB2* promoter regions in the wild type (BY2125) (lanes 1 and 4), *cln3ecb-5* (BY2278) (lanes 2 and 5), and *cln3ecb-6* (BY2690) (lanes 3 and 6) mutant strains. Cells were grown in YEP galactose at 30°C, and CHIPs were performed using Mcm1-specific antibodies. Lane 7 shows PCR products obtained with yeast genomic DNA as the template. *CLN3*–ECB-a probe includes the first four potential ECB elements, and *CLN3*–ECB-b includes the fifth and sixth such sites.

also carried out with yeast crude extracts and identical results were obtained (data not shown).

In vivo binding of Mcm1 to ECB elements. To measure in vivo association of Mcm1 with ECB-containing promoters, CHIP was used (15). Live cells were treated with formaldehyde to cross-link tightly associated proteins to their DNA binding sites. Then DNA cross-linked to Mcm1 was immunoprecipitated using a polyclonal Mcm1-specific antibody. The abundance of specific promoter sequences within these anti-Mcm1 immunoprecipitates was analyzed using PCR. Each reaction contained several primer pairs, which enabled us to detect several DNA fragments simultaneously. Figure 7C shows that the *CLN3*–ECB-1-4 region and *SWI4*-ECB and *CLB2*-ECB sequences are preferentially immunoprecipitated when cross-linked DNA is prepared from logarithmically growing cells. The precipitate is specific to the Mcm1 antibody, and a control sequence (*ACT1*) is not cross-linked at all (Fig. 7C, lanes 2 and

3). *CLN3*-ECB and *SWI4*-ECB sequences can also be detected in precipitates generated without cross-linking (Fig. 7C, lane 1), suggesting that the binding of Mcm1 to these sequences is strong and that the dissociation rate is slow.

In all the CHIP assays performed, the *CLN3*–ECB-1-4 region was the preferred binding sequence (*CLN3*–ECB-a probe in Fig. 7). Although in vivo association of Mcm1 to the *CLN3*–ECB-5-6 region covered by the *CLN3*–ECB-b probe can be detected (Fig. 7D, lane 4) binding of Mcm1 to this part of the promoter is very weak. However, when we repeated the CHIP analysis with strains carrying mutations in the first five ECBs (Fig. 7D, lane 5) or all six potential chromosomal ECB sequences (Fig. 7D, lane 6), it was evident that the presence of ECB6 alone is not sufficient to detect Mcm1 binding. This is
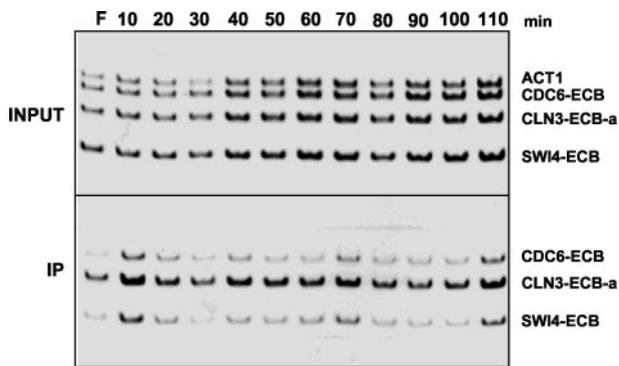
FIG. 8. Chromatin association of Mcm1 through the cell cycle. Wild-type cells grown in glucose were synchronized using α-factor. After 90 min, α-factor was removed by filtration (7) and cells were released into fresh media. Samples were taken at the end of the arrest (lane αF) and every 10 min for 110 min after the release. CHIPs were performed for each sample and assayed for the presence of *ACT1*, *CDC6*, *CLN3*, and *SWI4* promoter sequences. The top half shows PCR products obtained from chromatin extracts before immunoprecipitation with Mcm1 antibody (INPUT). The bottom half shows PCR products obtained with immunoprecipitated DNA (IP).

not surprising because *CLN3*-ECB6 does not have a good ECB consensus (Fig. 7B) and is only weakly bound in vitro as measured by DNase I footprinting (Fig. 2). Our in vivo analysis of mutations of the putative *CLN3* ECBs also indicates that the sixth putative ECB has no impact upon the transcriptional activity of the *CLN3* promoter (28).

Mcm1 is associated with ECB elements throughout cell cycle. To address whether the association of Mcm1 with ECB-containing promoters changes through the cell cycle, CHIP assays were performed with α-factor-synchronized cultures. Binding of Mcm1 to the ECB-containing promoters of *CDC6*, *CLN3*, and *SWI4* could be detected throughout the cell cycle (Fig. 8). As seen in steady-state measurements (Fig. 7), the strongest binding of Mcm1 throughout the cell cycle is observed to the *CLN3*-ECB-a region. This is not due to a preferred amplification of this promoter fragment in a reaction containing several primer pairs, because the same result was obtained when the reaction was done using only one pair of primers (data not shown). A quantification of the result represented in Fig. 8 showed less than twofold fluctuations through the cell cycle, although there was generally less binding observed in α-factor-arrested cultures (Fig. 8, first lane). A qualitatively similar result was obtained with cells synchronized by heat inactivation of a temperature-sensitive *cdc15* allele (data not shown). In both experiments, the Mcm1/DNA complex was detectable throughout the cell cycle and there was no systematic variation consistent with periodic occupation of the ECB sites. From this we conclude that the regulation of ECB activity is not achieved through changes in Mcm1 binding to DNA.

ECB binding complexes are affected by carbon source changes. Several studies have suggested that Mcm1 activity may be sensitive to carbon source changes (4, 8). To see if ECB binding was also affected, we carried out CHIP analysis with cells grown in rich YEP medium supplemented with 2% glucose, galactose, raffinose, or glycerol (Fig. 9A). As before, the *CLN3*–ECB-a probe shows the strongest binding and this bind-

ing is the least affected by the change of carbon source. However, all three ECB probes show the same tendency to increased binding in the poor carbon sources (raffinose and glycerol). Interestingly, we see an inverse correlation between binding of Mcm1 in vivo, as assayed by CHIP analysis, and the level of *CLN3* message attained. There is more Mcm1 bound, but there is two- to threefold less *CLN3* message accumulation in the poor carbon sources than in glucose (Fig. 9B). This may reflect the influence of other promoter elements (e.g., the GREs found in the *CLN3* promoter) (37, 38); however, we see a similar drop in transcription using isolated ECB elements driving *lacZ* expression (data not shown). Western analysis shows that more Mcm1 is produced in cells growing in glucose as a carbon source than in glycerol-grown cells (Fig. 9C). This is also reflected in the ability to form more Mcm1/ECB complexes in vitro, as assayed by band shift analysis (Fig. 9D). These data suggest that the Mcm1 may be bound in an inactive complex to ECBs in cells grown in poor carbon sources.

DISCUSSION

Mcm1 is required for the expression of many constitutively transcribed genes and a subset of the M- and M/G$_1$-specific transcripts. As a result, some fraction of the Mcm1 in a cell must be present in the nucleus and functional throughout the cell cycle. The restriction of its activity to either M or the M/G$_1$ transition must be determined by the promoter context in which it binds. The factors which interact with Mcm1 and confer M-specific transcription have been identified recently (17, 23, 25, 27, 41, 54). In this paper we provide evidence that the M/G$_1$-specific and Mcm1-dependent complexes that form on ECB elements are also large and heterogeneous and contain at least one other protein. Moreover, we identify sequences flanking the Mcm1 binding site that affect the binding and activity of these complexes. We also characterize the binding of Mcm1 to ECBs in vivo during the cell cycle and in different carbon sources.

The Mcm1 binding site has been exhaustively studied. Site selection identified the minimal 10-bp sequence required for Mcm1 binding in vitro (34). In addition, the larger 16-bp palindromic sequence that is required for function in vivo has been mutagenized at every position (1) and the crystal structure of an Mcm1 fragment/Matα2/DNA complex has been determined (48). As a result, the bases within the 16-bp palindrome that make contact with the Mcm1 and are required for Mcm1 binding and activity are known in at least a few contexts. In early studies, it was noted that the α-specific genes that are induced by Mcm1 and α1 show a striking divergence from the canonical Mcm1 binding site on the side adjacent to the α1 binding site and that the presence of α1 provides necessary stability to these complexes (21). Thus, the degenerate Mcm1 binding site serves to make Mcm1 binding and activation of these promoters dependent upon the accessory factor α1, which is only present in α cells. In the case of the M-specific transcripts, many of the Mcm1 binding sites contain noncanonical residues on the side opposite to that to which the Fkh proteins bind. However, in the few cases tested, Fkh binding requires the presence of Mcm1 (2, 25) and both are bound constitutively through the cell cycle (23). Activation requires
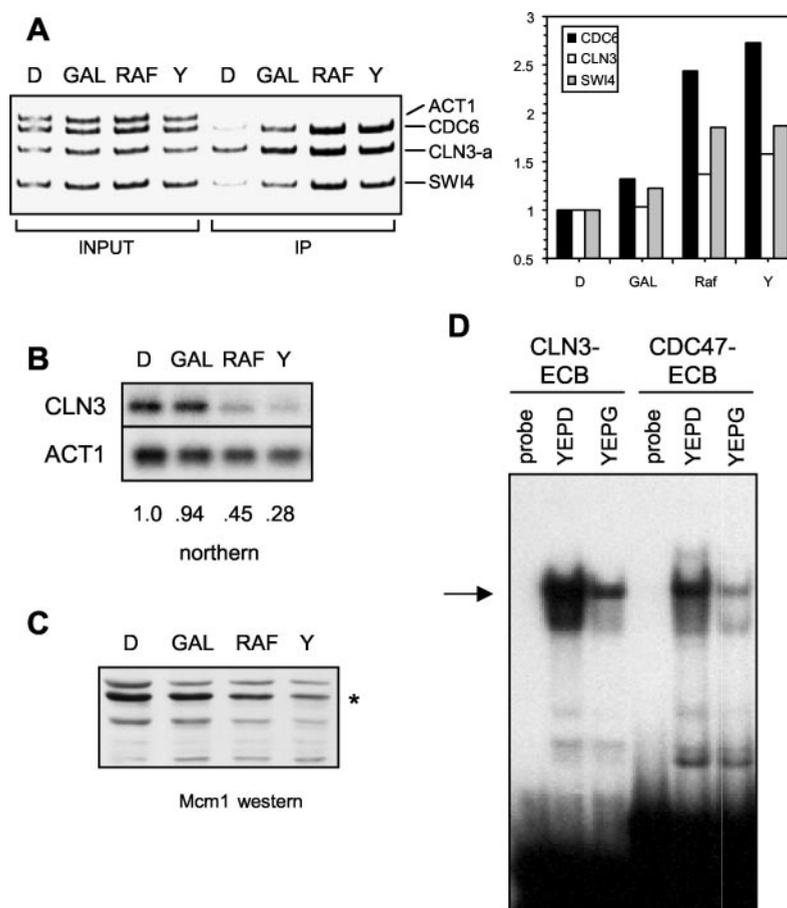
FIG. 9. Mcm1-ECB interaction in different carbon sources. (A) Wild-type cells were grown in YEP medium supplemented with 2% glucose (D), galactose (gal), raffinose (raf), or glycerol (Y), and CHIP analysis was performed as described for Fig. 7. At right is shown the data quantified as ratios of the amount immunoprecipitated (IP) over the input and normalized to the value obtained in the glucose-grown cells. (B) Northern blot analysis of cultures used for the CHIP experiments. The blot was sequentially probed for *CLN3* and *ACT1* and quantified. The ratio of counts in *CLN3* over *ACT1* is shown below each lane. Because the *ACT1* transcript is also lower in cells grown in the poor carbon sources, this value is an underestimate of the reduction of *CLN3* mRNA under these conditions. (C) Western analysis detecting Mcm1 protein (asterisk) from extracts of cells grown in the carbon sources indicated. (D) Gel retardation assays using crude extracts from wild-type cells grown in YEP medium supplemented with 2% glucose (YEPD) or 2% glycerol (YEPG) and ECB-containing probes from the *CLN3* or *CDC47* promoter. The arrow depicts the position of the Mcm1-specific protein-DNA complex, and the first and fourth lanes show the migration of probe alone (probe).

the cell-cycle-regulated association of a third protein called Ndd1 (23, 27).

The **a**-specific promoter elements show a high degree of conservation across the 16-bp palindrome. In addition, there are symmetrically placed binding sites for the homeobox protein α2, which represses these genes in α cells. Activation of these genes in **a** cells requires the Ste12 protein (12, 14). Unlike the other Mcm1 accessory proteins, Ste12 binding sites are not necessarily adjacent to the Mcm1 site; rather, they are often found in multiple copies and at variable distances from the Mcm1 binding site (24). The best studied of the **a**-specific genes is *STE2*. Mcm1 can bind the *STE2* element in the absence of Ste12, but it only weakly activates transcription (18). This suggests that Mcm1 cannot activate transcription on its own; rather, it relies on associated proteins that confer this property to the complex. Interestingly, some **a**-specific genes (including *STE2*) are also cell cycle regulated and peak at the M/G$_1$ boundary (36), so they may have some regulatory elements in common with the ECB-regulated genes.

Alignment of the M/G$_1$-specific promoter elements shows that they are symmetrically conserved across the palindrome, but little other sequence conservation is evident. Based upon the similarity between the Mcm1 binding sites in the M/G$_1$-specific genes and the other well-studied sites, we expected that Mcm1 could bind these sites in the absence of accessory factors. Consistent with this, we have shown that in vitro-translated Mcm1 binds to these elements (Fig. 4 and data not shown). Moreover, Mcm1 is bound to ECB elements throughout the cell cycle. So, just as with the M-specific and **a**-specific genes, the binding of Mcm1 to the promoter element is not sufficient to activate transcription. Other proteins or modifications of Mcm1 activity must be involved, and the DNA context of the ECB element must be responsible for their specification as M/G$_1$-specific transcription elements.

The additional sequence information restricting ECB activity to the M/G$_1$ boundary of the cell cycle could be distal to the Mcm1 binding site, as is the case with Ste12, or it could be embedded within it. In the case of *CDC6*, both distal and

proximal sequence elements may be in play because there is at least one Swi5 binding site near the fourth ECB element (11, 49). Swi5 is required for maximal transcription of this M/G$_1$-specific gene (40), but Swi5 is also required for transcription of genes, like *HO*, which are expressed at a later stage of the cell cycle (6, 45). At the *HO* promoter, Swi5 has been shown to recruit chromatin remodeling factors that in turn enable the late G$_1$-specific transcription factors, Swi4 and Swi6, to bind and activate transcription at distal SCB elements (10). Swi5 may act in an analogous fashion at a subset of the M/G$_1$-specific promoters. However, Swi5 is not responsible for the cell cycle specificity of ECB elements, because we have shown that small DNA fragments including the two tandem ECBs from *CDC47* or the single *SWI4* ECB, cloned into a *lacZ* reporter construct, are sufficient to confer M/G$_1$-specific transcription (32). Neither of these constructs includes a Swi5 binding site, so there is no reason to think that Swi5 is involved. Rather, the sequence information required to restrict ECB activity to the M/G$_1$ boundary is likeliest to be embedded within the 16-bp palindrome.

In order to identify the *cis-* and *trans-*activators of ECB elements, we have carried out a series of experiments. Simple alignment of the elements shows that, in addition to maintaining preferred residues for Mcm1 binding within the palindrome, there is further conservation extending a few bases beyond the palindrome and at positions -3 and $+3$ within the palindrome, where mutagenesis and crystallographic studies indicate that Mcm1 should have no base-specific contacts (1, 48). Not all putative ECBs contain these additional conserved residues, but all but one of the M/G$_1$-specific promoters under study contain more than one putative ECB. Thus, we do not know which of these sites are active. It could be that M/G$_1$-specific regulation involves binding of another protein to a subset of these sites via conserved bases adjacent to and/or embedded within the otherwise palindromic Mcm1 binding site.

Our studies of the ECB binding complex verify the importance of Mcm1 in ECB activation, but they also reveal additional complexities that are indicative of the presence of other proteins in the ECB complex. DNase I protection studies showed that all four of the M/G$_1$-specific promoters analyzed have complex patterns of protection of the ECB elements extending about 10 bp on one or both sides of the palindrome to which the Mcm1 dimer is known to bind. Gel retardation assays show that ECB complexes from crude cell extracts are highly heterogeneous compared to those formed with in vitro-translated Mcm1. Moreover, the nature and stability of ECB complexes are influenced by the sequence of the flanking DNA. Mutation of the flanking sequences from the fourth *CLN3* ECB results in a threefold-higher dissociation constant for the binding complexes and reduces the variety of complexes that can be formed on the ECB.

The bases critical for complex formation and stability have not been exhaustively analyzed; however, we have noted a region of limited homology in the protected flanking region and shown that substitutions at the most conserved positions affect transcriptional activity of the ECB element in vivo. Cell cycle regulation persists in spite of these changes, but the activity is elevated and possibly extended for a broader interval of time. This suggests that the flanking sequence may affect the stability, rather than the composition, of the complexes that form on ECB elements through the cell cycle. The possibility that flanking sequences influence the activity of the ECB under specific environmental conditions (e.g., carbon source shifts) is being investigated.

Gel filtration shows that the binding complex on ECB elements is in excess of 200 kDa. This is far larger than expected for a dimer of Mcm1, which would be 70 kDa, so it is likely that other proteins are associated with the complex. In fact, the dimeric and monomeric forms of Mcm1 are not detectable in the gel filtration fractions. This indicates that most of the Mcm1 in the cell is associated with other proteins. The large complex which binds ECB elements in this assay lacks at least one protein of 25 kDa, as that size fraction must be added back to generate a band shift complex of wild-type mobility. We have assayed the formation of the ECB-specific band shift complex from extracts of cells deleted for the DNA binding proteins Swi5 (40), the related protein Ace2 (31), and Ste12 (12, 14). None of these proteins appears to be involved, as the behavior of the Mcm1-specific complex did not change (data not shown).

Mcm1 binding to ECB elements does not change through the cell cycle, but it is affected by changes in the carbon source. CHIP analysis shows that Mcm1 binding complexes on ECBs from the *CLN3*, *CDC6*, and *SWI4* promoters are much more prevalent in cells grown on poor carbon sources like glycerol and raffinose than they are in glucose-grown cells. In spite of the increased binding, ECB elements are less active in poor carbon sources. This suggests that an inactive form of the complex is being stabilized on the ECB under nonoptimal growth conditions. *PIS1*, another Mcm1-regulated gene, is also down-regulated in poor carbon sources (4), so this may be a general property of Mcm1. Chen and Tye have shown that the activity of unstable alleles of *MCM1* can be enhanced by reduced glycolytic flux (8), but the signaling metabolite has not been identified. Mcm1 activity is also affected by osmotic stress and perhaps by other environmental changes mediated by the Sln1 two-component response regulator (50, 51). Modification of Mcm1 by phosphorylation has also been detected during salt stress (26).

Further studies are required to understand the dynamics of Mcm1 activity at ECB elements through the cell cycle and in response to environmental cues. The products of the ECB-regulated genes under study either promote the G$_1$-to-S transition (*SWI4* and *CLN3*) or are involved in the formation of prereplication complexes at which DNA synthesis is initiated (*CDC47* and *CDC6*). Understanding how internal and external signals influence the expression of these genes may provide new insights into the control of the early events of the cell cycle.

REFERENCES

1. **Acton, T. B., H. Zhong, and A. K. Vershon.** 1997. DNA-binding specificity of Mcm1: operator mutations that alter DNA-binding and transcriptional ac-

tivities by a MADS box protein. Mol. Cell. Biol. **17:**1881–1889.

2. **Althoefer, H., A. Schleiffer, K. Wassmann, A. Nordheim, and G. Ammerer.** 1995. Mcm1 is required to coordinate G$_2$-specific transcription in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **15:**5917–5928.

3. **Ammerer, G.** 1990. Identification, purification, and cloning of a polypeptide (PRTF/GRM) that binds to mating-specific promoter elements in yeast. Genes Dev. **4:**299–312.

4. **Anderson, M. S., and J. M. Lopes.** 1996. Carbon source regulation of *PIS1* gene expression in *Saccharomyces cerevisiae* involves the *MCM1* gene and the two-component regulatory gene, *SLN1.* J. Biol. Chem. **271:**26596–26601.

5. **Breeden, L., and G. Mikesell.** 1991. Cell cycle-specific expression of the SWI4 transcription factor is required for the cell cycle regulation of *HO* transcription. Genes Dev. **5:**1183–1190.

6. **Breeden, L., and K. Nasmyth.** 1987. Cell cycle control of the yeast *HO* gene: *cis*- and *trans*-acting regulators. Cell **48:**389–397.

7. **Breeden, L. L.** 1997. Alpha factor synchronization of budding yeast. Methods Enzymol. **283:**332–341.

8. **Chen, Y., and B.-K. Tye.** 1995. The yeast Mcm1 protein is regulated post-transcriptionally by the flux of glycolysis. Mol. Cell. Biol. **15:**4631–4639.

9. **Cocker, J. H., S. Piatti, C. Santocanale, K. Nasmyth, and J. F. X. Diffley.** 1996. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. Nature **379:**180–182.

10. **Cosma, M. P., T. Tanaka, and K. Nasmyth.** 1999. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally-regulated promoter. Cell **97:**299–311.

11. **Dohrmann, P. R., G. Butler, K. Tamai, S. Dorland, J. R. Greene, D. J. Thiele, and D. J. Stillman.** 1992. Parallel pathways of gene regulation: the homologous regulators *SWI5* and *ACE2* differentially control transcription of *HO* and chitinase. Genes Dev. **6:**93–104.

12. **Dolan, J. W., C. Kirkman, and S. Fields.** 1989. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Natl. Acad. Sci. USA **86:**5703–5707.

13. **Dubois, E., and F. Messenguy.** 1991. In vitro studies of the binding of the ARGR proteins to the *ARG5,6* promoter. Mol. Cell. Biol. **11:**2162–2168.

14. **Errede, B., and G. Ammerer.** 1989. STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. Genes Dev. **3:**1349–1361.

15. **Hecht, A., S. Strahl-Bolsinger, and M. Grunstein.** 1996. Spreading of transcriptional repressor Sir3 from telomeric heterochromatin. Nature **383:**92–96.

16. **Herschbach, B., M. Arnaud, and A. Johnson.** 1994. Transcriptional repression directed by the yeast alpha 2 protein in vitro. Nature **370:**309–311.

17. **Hollenhorst, P. C., M. E. Bose, M. R. Mielke, U. Muller, and C. A. Fox.** 2000. Forkhead genes in transcriptional silencing, cell morphology and the cell cycle: overlapping and distinct functions for *FKH1* and *FKH2* in *Saccharomyces cerevisiae.* Genetics **154:**1533–1548.

18. **Hwang-Shum, J.-J., D. C. Hagen, E. E. Jarvis, C. A. Westby, and G. F. Sprague.** 1991. Relative contributions of MCM1 and STE12 to transcriptional activation of a- and α-specific genes from *Saccharomyces cerevisiae.* Mol. Gen. Genet. **227:**197–204.

19. **Iyer, V., and K. Struhl.** 1996. Absolute mRNA levels and transcriptional initiation rates in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **93:**5208–5212.

20. **Jarvis, E. E., K. L. Clark, and G. F. Sprague.** 1989. The yeast transcription activator PRTF, a homolog of the mammalian serum response factor, is encoded by the *MCM1* gene. Genes Dev. **3:**936–945.

21. **Jarvis, E. E., D. C. Hagen, and G. F. Sprague, Jr.** 1988. Identification of a DNA segment that is necessary and sufficient for a α-specific gene control in *Saccharomyces cerevisiae*: implications for regulation of **a**-specific and α-specific genes. Mol. Cell. Biol. **8:**309–320.

22. **Keheler, C. S., A. S. Passmore, and A. D. Johnson.** 1989. Yeast repressor α2 binds to its operator cooperatively with yeast protein Mcm1. Mol. Cell. Biol. **9:**5228–5230.

23. **Koranda, M., A. Schleiffer, L. Endler, and G. Ammerer.** 2000. Forkhead-like transcription factors recruit Ndd1 to the chromatin of G2/M-specific promoters. Nature **406:**94–97.

24. **Kronstad, J. W., J. A. Holly, and V. L. MacKay.** 1987. A yeast operator overlaps an upstream activation site. Cell **50:**369–377.

25. **Kumar, R., D. M. Reynolds, A. Shevchenko, A. Shevchenko, S. D. Goldstone, and S. Dalton.** 2000. Forkhead transcription factors, Fkh1p and Fkh2p, collaborate with Mcm1p to control transcription required for M-phase. Curr. Biol. **10:**896–906.

26. **Kuo, M.-H., E. T. Nadeau, and E. J. Grayhack.** 1997. Multiple phosphorylated forms of the *Saccharomyces cerevisiae* Mcm1 protein include an isoform induced in response to high salt concentrations. Mol. Cell. Biol. **17:**819–832.

27. **Loy, C. J., D. Lydall, and U. Surana.** 1999. *NDD1*, a high-dosage suppressor of *cdc28-1N*, is essential for expression of a subset of late-S-phase-specific genes in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **19:**3312–3327.

28. **MacKay, V. L., B. Mai, L. Waters, and L. L. Breeden.** 2001. Early cell cycle box-mediated transcription of *CLN3* and *SWI4* contributes to the proper timing of the G$_1$-to-S transition in budding yeast. Mol. Cell. Biol. **21:**4140–4148.

29. **Mai, B., and L. Breeden.** 1997. Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family. Mol. Cell. Biol. **17:**6491–6501.

30. **McBride, H. J., R. M. Brazas, Y. Yu, K. Nasmyth, and D. J. Stillman.** 1997. Long-range interactions at the *HO* promoter. Mol. Cell. Biol. **17:**2669–2678.

31. **McBride, H. J., Y. Yu, and D. J. Stillman.** 1999. Distinct regions of the Swi5 and Ace2 transcription factors are required for specific gene activation. J. Biol. Chem. **274:**21029–21036.

32. **McInerny, C. J., J. F. Partridge, G. E. Mikesell, D. P. Creemer, and L. L. Breeden.** 1997. A novel Mcm1-dependent promoter element in the *SWI4*, *CLN3*, *CDC6* and *CDC47* promoters activates M/G1-specific transcription. Genes Dev. **11:**1277–1288.

33. **Messenguy, F., and E. Dubois.** 1993. Genetic evidence for a role for MCM1 in the regulation of arginine metabolism in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **13:**2586–2592.

34. **Nurrish, S. J., and R. Treisman.** 1995. DNA binding specificity determinants in MADS-box transcription factors. Mol. Cell. Biol. **15:**4076–4085.

35. **Odom, A. R., A. Stahlberg, S. R. Wente, and J. D. York.** 2000. A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science **287:**2026–2029.

36. **Oehlen, L. J. W. M., J. D. McKinney, and F. R. Cross.** 1996. Ste12 and Mcm1 regulate cell cycle-dependent transcription of *FAR1.* Mol. Cell. Biol. **16:**2830–2837.

37. **Parviz, F., D. D. Hall, D. D. Markwardt, and W. Heideman.** 1998. Transcriptional regulation of *CLN3* expression by glucose in *Saccharomyces cerevisiae.* J. Bacteriol. **180:**4508–4515.

38. **Parviz, F., and W. Heideman.** 1998. Growth-independent regulation of *CLN3* mRNA levels by nutrients in *Saccharomyces cerevisiae.* J. Bacteriol. **180:**225–230.

39. **Passmore, S., R. Elble, and B.-K. Tye.** 1989. A protein involved in minichromosome maintenance in yeast binds a transcriptional enhancer conserved in eukaryotes. Genes Dev. **3:**921–935.

40. **Piatti, S., C. Lengauer, and K. Nasmyth.** 1995. Cdc6 is an unstable protein whose *de novo* synthesis in G$_1$ is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae.* EMBO J. **14:**3788–3799.

41. **Pic, A., F.-L. Lim, S. J. Ross, E. A. Veal, A. L. Johnson, M. R. A. Sultan, A. G. West, L. H. Johnston, A. D. Sharrocks, and B. A. Morgan.** 2000. The forkhead protein Fkh2 is a component of the yeast cell cycle transcription factor SFF. EMBO J. **19:**1–12.

42. **Shore, P., and A. D. Sharrocks.** 1995. The MADS-box family of transcription factors. Eur. J. Biochem. **229:**1–13.

43. **Sprague, G. F., Jr., R. Jensen, and I. Herskowitz.** 1983. Control of yeast cell type by the mating type locus: positive regulation of the α-specific STE3 gene by the MATα1 product. Cell **32:**409–415.

44. **Sprague, G. F. J.** 1990. Combinatorial associations of regulatory proteins and the control of cell type in yeast. Adv. Genet. **27:**33–62.

45. **Stern, M., R. Jensen, and I. Herskowitz.** 1984. Five *SWI* genes are required for expression of the *HO* gene in yeast. J. Mol. Biol. **178:**853–868.

46. **Stillman, B.** 1996. Cell cycle control of DNA replication. Science **274:**1659–1664.

47. **Strahl-Bolsinger, S., A. Hecht, K. Luo, and M. Grunstein.** 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. **11:**83–93.

48. **Tan, S., and T. J. Richmond.** 1998. Crystal structure of the yeast MATα2/MCM1/DNA ternary complex. Nature **391:**660–666.

49. **Tebb, G., T. Moll, C. Dowzer, and K. Nasmyth.** 1993. SWI5 instability may be necessary but is not sufficient for asymmetric HO expression in yeast. Genes Dev. **7:**517–528.

50. **Yu, G., R. J. Deschenes, and J. S. Fassler.** 1995. The essential transcription factor, Mcm1, is a downstream target of Sln1, a yeast "two-component" regulator. J. Biol. Chem. **270:**8739–8743.

51. **Yu, G., and J. S. Fassler.** 1993. SPT13 (GAL11) of *Saccharomyces cerevisiae* negatively regulates activity of the MCM1 transcription factor in Ty1 elements. Mol. Cell. Biol. **13:**63–71.

52. **Zhong, H., R. McCord, and A. K. Vershon.** 1999. Identification of target sites of the α2-Mcm1 repressor complex in the yeast genome. Genome Res. **9:**1040–1047.

53. **Zhou, C., and A. Y. Jong.** 1993. Mutation analysis of *Saccharomyces cerevisiae* *CDC6* promoter: defining its UAS domain and cell cycle regulating element. DNA Cell Biol. **4:**363–370.

54. **Zhu, G., P. T. Spellman, T. Volpe, P. O. Brown, D. Botstein, T. N. Davis, and B. Futcher.** 2000. Two yeast forkhead genes regulate the cell cycle and pseudohyphal growth. Nature **406:**90–93.